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SHORT COMMUNICATION

The Pit-1 Transcription Factor Gene Is a Candidate for the Murine Snell Dwarf Mutation

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Two nonallelic mouse mutations with severe dwarf phenotypes are characterized by a lack of growth hormone, prolactin, and thyroid stimulating hormone. The cells that normally synthesize these pituitary hormones express a common transcription factor called GHF-1 or Pit-1. Using an intersubspecific backcross, we have demonstrated tight linkage of the *Pit-1* and Snell dwarf (*dw*) genes on mouse chromosome 16. No recombination was observed between *Pit-1* and *dw* in 110 individuals examined. Southern blot analysis of genomic DNA reveals that the *Pit-1* gene is rearranged in C3H/HeJ-*dw*^{J}/*dw* mice but not in coisogenic +/+ animals, providing molecular evidence that a lesion in the *Pit-1* gene results in the Snell dwarf phenotype. Demonstration of low levels of *Pit-1* expression in Ames dwarf (*df*) mice implies that both *Pit-1* and *df* expression may be required for pituitary differentiation.

Two nonallelic, autosomal recessive mutations in mice are characterized by pituitary defects leading to severe dwarfism. Mice homozygous for the Snell (*dw*) and Ames (*df*) dwarf mutations are indistinguishable from heterozygotes at birth, but adult homozygous animals are about one-third normal size. Both mutants are characterized by diminished anterior pituitary function. Growth hormone (GH), prolactin (PRL), and thyroid stimulating hormone (TSH) are not detectable, nor are their corresponding cell types, somatotrophs, lactotrophs, and thyrotrophs (Bartke, 1964, 1979; Roux *et al.*, 1982; Cheng *et al.*, 1983). The lack of detectable PRL and GH at any time during development indicates that both mutations result in a failure to initiate synthesis of these hormones and suggests that these mutants provide models of blocked differentiation (Slabaugh *et al.*, 1982).

A transcription factor that is normally found in the three cell types that are absent from both dwarf mutants has been isolated. This transcription factor, called Pit-1 or GHF-1, was cloned on the basis of specific binding to the promoters of the GH and PRL genes and the PRL enhancer (Bodner *et al.*, 1988; Ingraham *et al.*, 1988). A variety of functional assays have shown that Pit-1 plays a role in transcription of the GH and PRL genes, although the importance of Pit-1 for prolactin transcription is still controversial (Ingraham *et al.*, 1988; Mangalam *et al.*, 1989; Theill *et al.*, 1989; Karin *et al.*, 1990; Dolle *et al.*, 1990). It is not clear whether Pit-1 is involved in transcriptional activation of the β subunit of TSH, but it is found in some thyrotrophs (Ingraham *et al.*, 1988; Alexander *et al.*, 1989). The factor is an excellent candidate for one of the dwarf lesions because it is specific to the three cell types affected in the *df* and *dw* dwarfs, and it is known to be a transcription factor for two of the three missing hormones.

A mouse *Pit-1* cDNA clone was prepared by reverse transcription of pituitary mRNA followed by polymerase chain reaction (PCR) amplification (Krug and Berger, 1987; Saiki *et al.*, 1988). Poly(A)$^{+}$mRNA was prepared from pituitaries of C57BL/6J × SJL/J F2 mice, and oligo(dT)-primed synthesis of cDNA using reverse transcriptase was performed. Oligonucleotides for PCR were synthesized on the basis of the rat *Pit-1* nucleotide sequence (Ingraham *et al.*, 1988) using regions of *Pit-1* that are conserved between rat and bovine (Bodner *et al.*, 1988) (nucleotides 139–167 and 778–807) with the incorporation of *Hin*dIII and *Bam*HI linkers to facilitate subsequent cloning. The 689-bp amplification product, corresponding to amino acid residues 47–269, was cloned into the *Hin*dIII and *Bam*HI sites of pBluescript KS- (Strata-

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gene). The identity of the cDNA was confirmed by dideoxy DNA sequence analysis (Sanger *et al.*, 1977).

Two backcrosses were used to determine whether *Pit-1* is associated with either dwarf mutation. Laboratory strains carrying the *df* or *dw* mutations were crossed to inbred strains derived from the *Mus musculus* subspecies *castaneus* (CASA/Rk) or *molossinus* (MOLD/Rk). The evolutionary distance between subspecies greatly facilitates identification of restriction fragment length polymorphisms (RFLPs) (Reeves *et al.*, 1987). The restriction enzyme *Bam*HI detected an RFLP at the *Pit-1* locus between CASA/Rk mice and the inbred strain carrying the Ames *df* mutation (referred to herein as DF) (Fig. 1A). DNA samples from 32 progeny of the backcross (CASA/RK × DF *df/df*) × DF *df/df* were analyzed with the *Pit-1* probe after digestion with *Bam*HI. Restriction patterns for 12 individuals are presented in Fig. 1A. Recombination between *Pit-1* and *df* was observed in 16 of the 32 animals examined. The independent assortment of the *Pit-1* and *df* genes demonstrates that they are different, unlinked loci. Linkage of *Pit-1* with *dw* was then tested on the backcross (MOLD/Rk × DW/J *dw/dw*) × DW/J *dw/dw*. Eighty-three progeny of this cross have been typed for 10 markers which cover most of chromosome 16 (O'Hara *et al.*, 1988; Reeves *et al.*, submitted). An RFLP detected by digestion with *Pvu*II was used to map *Pit-1* between *Gap43* and *D21S16h*, to the same position as *dw* (Table 1 and Fig. 1B). Twenty-seven additional animals were typed for *dw* and *Pit-1*. No recombination was observed in any of the 110 animals typed, indicating with 95% confidence that *dw* and *Pit-1* are separated by no more than 2.7 cM.

The *dw* mutation has arisen twice. The first isolation occurred in silver mice in 1929 and is carried in the inbred DW/J strain (Snell, 1929). A second mutation, dw^J, arose spontaneously in the inbred C3H/HeJ strain at The Jackson Laboratory more than 40 years later (Eicher and Beamer, 1980). In an alleleism test, dwarf progeny resulted from mating mice heterozygous for the *dw* mutation with heterozygotes bearing the dw^J mutation, demonstrating that these are noncomplementing alleles of the same gene. To augment the genetic linkage of *Pit-1* and *dw* with molecular evidence that a mutation in the *Pit-1* gene is responsible for the *dw* mutation, congenic and coisogenic *dw/dw*, *dw/+*, and +/+ individuals carrying the *dw* and dw^J alleles were examined for evidence of a lesion in the *Pit-1* gene. Southern blot analysis of *Pit-1* using 28 restriction endonucleases revealed no differences between DW/J *dw/dw* and +/+, and most of the DW/J patterns examined were identical to those in C3H +/+. However, the restriction pattern of the C3H/HeJ-dw^J homozygotes was markedly different from that of coisogenic normal animals, with 12 of 14 restriction enzymes tested, indicating that the dw^J allele of the *Pit-1* gene is rearranged (Fig. 2). With *Hin*dIII and several other restriction enzymes (*Dra*I, *Xba*I, and *Pst*I; not shown) *dw/dw* individuals were shown to contain all of the *Pit-1*-related bands found in +/+ animals plus additional bands hybridizing to the *Pit-1* probe, indicating that the mutation responsible for dwarfism in C3H animals may involve a partial duplication of the *Pit-1* gene. Preliminary experiments with 5′ and 3′ specific probes, spanning amino

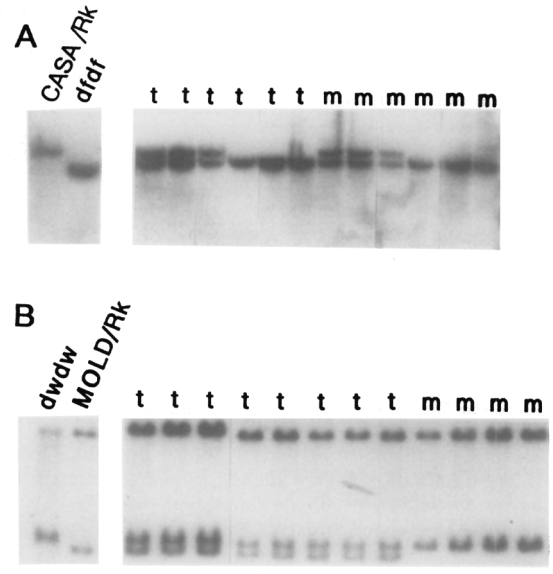

FIG. 1. Recombinational mapping of *Pit-1* in backcrosses segregating the *df* and *dw* mutations. (**A**) The *Pit-1* gene segregates independently of the Ames dwarf (*df*) mutation. DF mice were generously provided by Dr. Andrzej Bartke, S. Illinois University (Carbondale, IL). Male *df/df* weanlings were injected daily with thyroid hormone and ovine GH (22). Female dwarfs received thyroid hormone treatments followed by implantation of a pituitary from a normal female sib under the kidney capsule (13). After fertility was established, dwarf mice were maintained on AIN-76A diet containing 25 mg thyroid powder/kg diet (US Biochemicals) (11). CASA/Rk were from The Jackson Laboratory (Bar Harbor, ME). All mice were housed and cared for according to institutional guidelines (AAALAC approval No. A3114-01). Backcross progeny were scored as dwarf or normal based on facial characteristics and size at 4–5 weeks of age. For each individual, DNA was prepared from pooled spleen, kidneys, and lungs by phenol/chloroform extraction and ethanol precipitation. Following digestion with restriction endonucleases, fragments were separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with a *Pit-1* cDNA probe (22). Filters were then washed and autoradiographed for 1 to 4 days at −70°C with intensifying screens. DNAs from the inbred stocks are designated dfdf and CASA/Rk. Twelve backcross progeny that are heterozygous (t) or homozygous (m) for *df* are shown. (**B**) The *Pit-1* gene is tightly linked to the Snell dwarf (*dw*) mutation on mouse chromosome 16. DNA was prepared from the backcross segregating *dw* (22), digested with *Pvu*II, and analyzed by Southern blotting using the *Pit-1* probe. DNAs from the inbred stocks are designated dwdw and MOLD/Rk. Twelve backcross progeny that are heterozygous (t) or homozygous (m) for *dw* are shown.

TABLE 1

***Pit-1* Is Tightly Linked to *dw* on Chromosome 16**

Gap43	*dw*	*Pit-1*	*D21S16h*	Observed
m	m	m	m	32
t	t	t	t	31
m	m	m	t	2
t	t	t	m	5
m	t	t	t	8
t	m	m	m	5
Total				83

Gap43 to *dw/Pit-1*, 0.157 ± 0.040
dw/Pit-1 to *D21S16h*, 0.084 ± 0.031
Gap43 to *D21S16h*, 0.241 ± 0.047

Note. DNA samples from 83 progeny of the backcross (MOLD/Rk × DW/J *dw/dw*) × DW/J *dw/dw*, typed previously for 10 markers on chromosome 16, were analyzed to determine which *Pit-1* allele was inherited from the F_1 parent. Only the observed genotype classes are presented. Genes are shown in proximal to distal order. "m" indicates homozygosity for the DW/J allele; "t" indicates heterozygosity. Recombination frequencies are given ± standard error.

acids 47–100 and 205–269, respectively, were performed to verify that the lesion is within the *Pit-1* gene (not shown). The 5′ probe hybridized to the dw^J-specific bands in several instances (*Pvu*II, *Pst*I, and *Hinc*II digests), indicating that the lesion encompasses the 5′ portion of the gene. In contrast, the 3′ probe hybridized only to bands common to the mutant and wild-type alleles. The band specific for the mutant allele in the *Hin*dIII digest (Fig. 2) did not hybridize with either probe, suggesting that the dw^J lesion is within the *Pit-1* gene.

The different patterns of hybridization in DW/J and C3H/HeJ-dw^J dwarfs suggest that different molecular events disrupted Pit-1 function in the two *dw* alleles. We cannot rule out the possibility of a complex lesion in C3H/HeJ-dw^J which encompasses *Pit-1* as well as a neighboring gene. However, Pit-1 is specific to somatotroph, lactotroph, and thyrotroph cells and its expression is known to be sufficient to activate transcription of the GH and PRL genes in transfection studies (Ingraham *et al.*, 1988; Mangalam *et al.*, 1989). Thus, the available information about Pit-1 function and patterns of expression, together with the molecular and genetic data provided here, strongly suggests that disruption of the *Pit-1* gene results in the dwarf phenotype characteristic of the *dw* mutation. There are precedents for mutations in transcription factor genes that produce mutant phenotypes in mammals (Hughes *et al.*, 1988; Balling *et al.*, 1988).

The molecular basis for the Ames dwarf mutation (*df*) remains undefined. The *df* gene is unlikely to be the structural gene for any of the missing hormones. Its location on mouse chromosome 11 (Bartke, 1965; Camper and Katz, unpublished) eliminates the prolactin gene and the TSH α and β subunit genes as candidates (Jackson-Grusby *et al.*, 1988; Naylor *et al.*, 1983; Dracopoli *et al.*, 1988). The murine GH gene maps to mouse chromosome 11 (Jackson-Grusby *et al.*, 1988) but is located distal to the *df* gene (Camper and Katz, unpublished). Mutations in the GH gene would be expected to give rise to isolated GH deficiency, as is the case in humans and rats, rather than the panhypopituitarism of the mutant *df* mice (Phillips, 1985; Takeuchi *et al.*, 1990). By analogy to other mutants exhibiting a failure of multiple cell types (Chabot *et al.*, 1988; Geissler *et al.*, 1988; Jansson *et al.*, 1986), the lesion in Ames dwarf mice may result from a mutation in the gene for a growth factor or receptor. The *df* mutation might be expected to result in a failure to activate *Pit-1* expression, because transcription of GH and PRL promoters is activated by *Pit-1* expression in transfection studies (Ingraham *et al.*, 1988; Mangalam *et al.*, 1989). Pituitary RNA from *df/df* and *dw/dw* mice was examined for evidence of *Pit-1* expression. The extreme hypocellularity of the pituitaries in the mutant mice could be responsible for the lack of detectable expression on Northern blots (not shown). Therefore, a more sensitive PCR-based assay for *Pit-1* expression was employed (Fig. 3). Single-stranded cDNA was prepared from pituitary RNA and amplified by PCR with *Pit-1*-specific

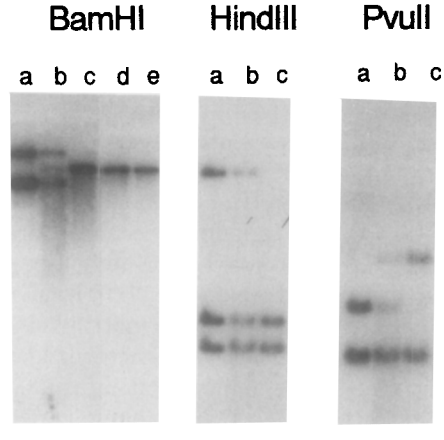

FIG. 2. Evidence for a lesion in the *Pit-1* gene. Rearrangement of the *Pit-1* gene was observed in C3H/HeJ-dw^J dwarf mice, but not in coisogenic +/+ animals or in DW/J +/+ or *dw/dw* mice. After digestion with the indicated restriction endonucleases, the *Pit-1* probe was hybridized to DNA from (**a**) C3H/HeJ-dw^J/dw^J; (**b**) C3H/HeJ-dw^J/+; (**c**) C3H/HeJ- +/+; (**d**) DW/J *dw/dw;* and (**e**) DW/J +/+ mice. DW/J mice were initially obtained from The Jackson Laboratory and maintained at the University of Michigan. C3H/HeJ-dw^J tissues were provided by Dr. Muriel Davisson, The Jackson Laboratory. To ensure that DNAs were completely digested, filters were stripped and rehybridized with a single-copy gene, granulocyte-macrophage colony stimulating factor (*Csfgm*) (32). There were no differences in the *Csfgm* patterns between the mutant and the wild-type animals (not shown).

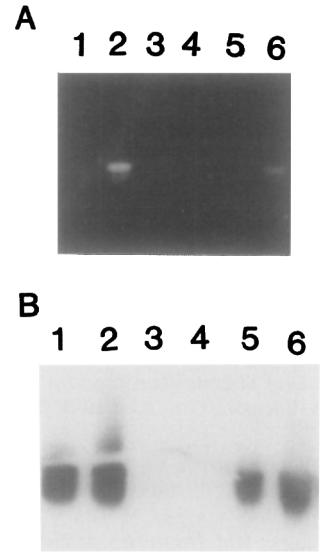

FIG. 3. *Pit-1* is expressed in both Ames and Snell dwarf pituitaries. Poly(A)$^+$ RNA was prepared from three to six pituitaries taken from dwarf mice homozygous for the *dw* (unrearranged) or *df* alleles and from normal littermates. The RNA samples were used as templates for oligo(dT)-primed cDNA synthesis. PCR amplification was performed with the same primers used to prepare the mouse *Pit-1* cDNA. The products were visualized by ethidium bromide staining after separation by agarose gel electrophoresis (**A**), transferred to a nylon filter, hybridized with a ^{32}P-labeled *Pit-1* oligonucleotide probe, and exposed to X-ray film for 2 h (**B**). The intensity of hybridization appears similar in the mutant and normal samples due to saturation of the DNA binding capacity of the filter. Lane **1** contains the amplification of *Pit-1* in pituitary cDNA from *dw/dw* mice; lane **2**, *dw/+*; lane **5**, *df/df*; and lane **6**, *df/+*. As negative controls, liver cDNA (lane **3**) and sham cDNA synthesized from tRNA (lane **4**) were amplified.

oligonucleotides. The amplification products were electrophoresed on an agarose gel and visualized by ethidium bromide staining. The expected 689-bp fragment was obvious in amplification of *dw/+* and *df/+* cDNA and faint, but detectable, in *dw/dw* and *df/df* cDNA. The sensitivity was further increased by probing a Southern blot of the amplification gel with the *Pit-1* cDNA. Pit-1 is clearly present in the *dw/dw* and *df/df* pituitary samples but absent from the liver and tRNA negative controls. This result was reproduced with independent preparations of pituitary mRNA, and the identity of the *Pit-1* amplification product confirmed by hybridization with an internal radiolabeled oligonucleotide spanning nucleotides 613 to 672. The presence of *Pit-1* mRNA in *df/df* pituitaries, which do not express GH or PRL, indicates that *Pit-1* expression alone is not sufficient for activation of GH and PRL expression *in vivo*, and implies that both the *df* gene product and *Pit-1* are required. The *df* backcross described here will be useful for testing for linkage of *df* with candidate genes such as growth factors, receptors, other pituitary-specific transcription factors and gene(s) involved in post-transcriptional regulation of *Pit-1* (Karin *et al.*, 1990; Dolle *et al.*, 1990).

Many of the consequences of the *df* and *dw* dwarf mutations can be reversed by administration of exogenous GH, PRL, and thyroxine, indicating that the dwarf phenotype arises primarily because of these hormone deficiencies. The hypocellularity of the dwarf pituitary is not affected by hormone replacement therapy; thus, *Pit-1* may be critical to the differentiation and maturation of somatotroph, lactotroph, and thyrotroph cells, since animals with an abnormal *Pit-1* gene (i.e., dw^J/dw^J animals) lack these cells. This contrasts with the hypothesis that *Pit-1* expression is critical only for somatotroph differentiation (Karin *et al.*, 1990; Dolle *et al.*, 1990). The *df* phenotype is very similar to that of *dw* mice, suggesting that the *df* mutation may disrupt a different step in the same developmental pathway. Moreover, the transcription of *Pit-1* in *df/df* pituitaries implies that both the *df* gene product and *Pit-1* may be required for differentiation of somatotrophs, lactotrophs, and thyrotrophs.

In conclusion, four criteria implicate the *Pit-1* gene as a candidate for the Snell dwarf mutation: genetic linkage, DNA rearrangement, appropriate expression patterns in wild-type mice, and reduced expression in the mutant. This suggests that the homeobox-containing transcription factor, Pit-1, is critical for differentiation of the three separate cell types lacking in the dwarf. Further analysis of the relationship between *df* and *dw* will provide significant insights into pituitary development and the cell specialization process.

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